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Analysis of expression of cyclin E, p27kip1 and Ki67 protein in colorectal cancer tissues and its value for diagnosis, treatment and prognosis of disease

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Abstract. – OBJECTIVE: We conducted this study is to investigate the clinical application value of Cyclin E, p27kip1 and Ki67 protein expression in colorectal cancer tissues for diagnosis, treatment, and prognosis of this disease.

PATIENTS AND METHODS: The positive expression of Cyclin E, p27kip1 and Ki-67 in tissues of 200 patients with colorectal cancer and 200 patients with benign colorectal tumor or inflammation were detected by immunohistochemistry PowerVision two-step method. RT-PCR was used to detect the expression level of the corresponding mRNA, as well as to analyze the association with TNM staging, pathology type, free progression survival and median survival. The sensitivity, specificity, and accuracy of diagnosis were analyzed by ROC.

RESULTS: The positive expression rate and positive degree of Cyclin E and Ki-67 of observation group were higher than those of the control group, while positive expression rate and positive degree of p27kipl was lower than that of the control group; the differences were statistically significant ($p<0.05$). The quantitative expression levels of Cyclin E and Ki-67 mRNA of observation group were distinctly higher than those of the control group, while p27kipl was evidently lower than that of the control group; the differences were statistically significant ($p<0.05$). With the increase of TNM staging, the positive expression of Cyclin E and Ki-67 increased, p27kipl decreased, and the difference was statistically significant ($p<0.05$). With the decrease of differentiation degree, the positive expression of Cyclin E and Ki-67 increased, p27kipl decreased, and the difference was statistically significant ($p<0.05$). The free progression survival and median survival of positive expression and negative expression of Cyclin E and Ki-67 were shortened, p27kipl extended, and the difference was statistically significant ($p<0.05$). The diagnostic sensitivity of Cyclin E mRNA was 89.6%, specificity 84.5%, accuracy 0.823 and the critical value was 0.3562; The diagnostic sensitivity of p27kipl mRNA was 80.5%, specificity 76.5%, accuracy 0.802 and the critical value was 0.3023. The diagnostic sensitivity of Ki-67 mRNA was 86.5%, specificity 82.9%, accuracy 0.814 and the critical value was 0.3243.

CONCLUSIONS: We discovered that Cyclin E and Ki67 protein expression of colorectal cancer tissues was upregulated and p27kipl protein expression was downregulated, which were closely related to the TNM and pathological differentiation degree. These values were also closely associated with free progression survival and median survival of prognosis. Therefore, the above indexes can be used as highly sensitive, specific and accurate markers for the diagnosis of colorectal cancer.

Key Words:
Colorectal cancer, Cyclin E, p27kipl, Ki-67, Immunohistochemistry PowerVision two-step method.

Introduction

The incidence of colorectal cancer ranks as the second most common malignant tumor in western developed countries and ranks third in the world only surpassed by the incidences of lung cancer and gastric cancer in male patients. In females, the incidence of colorectal cancer is only inferior to that of breast and cervical cancer[1]. In China, the incidence of colorectal cancer has been increasing gradually trending towards occurring in younger patients. While incidence and mortality of this cancer as ranks the fifth and sixth most common malignant tumor in China, new cases in the past five years ranks the third[2]. The development of colorectal cancer, which is widely accepted, is multi-step progression theory. Namely, it occurs due to the chromosomal instability pathway, which manifests that normal

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mucosa, undergoes the sequential evolution of hyperplasia, polyps, pre-invasive carcinoma and invasive carcinoma, leading to gradually accumulated genovariation[3,4]. Of those determinants, the disorders of cell cycle regulation mechanism are regarded as significant factors. The sequence conversion of the cell cycle is precisely regulated by signal transduction pathways and feedback loops, including cyclins, CDKs and CDKIs, of those, the regulation of CDKs is the central link[5]. This study indicated that Cyclin E is a positive regulation factor of CDKs, p27kipl is the significant member of CDKIs family, and Ki67 is the nuclear antigen, which is closely associated with mitosis[6]. Previous reports[7] have indicated that Cyclin E, p27kipl and Ki67 protein in colorectal cancer tissues have abnormal expression. This work is designed to analyze the application value of Cyclin E, p27kipl and Ki67 protein in diagnosis, treatment and prognosis of the disease.

Patients and Methods

Patients

200 cases of patients that were admitted to our hospital and diagnosed with colorectal cancer from January 2011 to January 2016 were continuously selected as the observation group, which was confirmed by histopathology. 200 cases of patients with benign colorectal tumor or inflammation were selected as the control group. Patients that had other tumors, colorectal metastatic neoplasm, infection, autoimmune disease, incomplete clinical data, and those did not follow-up were excluded. This study obtained the approval of the Ethics Committee in our hospital and gained the informed consent of patients and their families. In the observation group, there were 132 males and 68 females; their age was in the range of 38-77 years old, with the average as 52.6±14.7. There were 78 cases of colorectal cancer, and 122 cases of rectal carcinoma. According to TNM staging, there were 52 cases of stage I, 43 cases of Stage II, 60 cases of Stage III, and 45 cases of Stage IV cancer. Types of pathology included 58 cases of poorly differentiated adenocarcinoma, 72 cases of moderately differentiated adenocarcinoma, and 70 cases of highly differentiated adenocarcinoma. In the control group, there were 120 males and 80 females; the average age was 35-76 years old, with the average age being 52.7±16.9. There were 116 cases of polyps, and 84 cases of inflammation.

Methods

The positive expression of Cyclin E, p27kipl and Ki-67 in tissues was detected through immunohistochemistry Power Vision two-step method. The materials were imaged under the microscope according to the routine method to make tissue sections (thickness was 3 μm). Mouse anti-human monoclonal primary antibody and rabbit-mouse secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). DAB kit and two-step test kit were purchased from Beijing Zhongshan Jinqiao Biology (Co., Ltd., Beijing, China) with instructions to follow. Olympus microscope (Tokyo, Japan) camera, HMIAS-2000 full automatic color image analysis system (Invitrogen, Carlsbad, CA, USA) was used for analysis. The interpretation of results was according to the following guidelines: the nucleus or cytoplasm appeared brown yellow, which was regarded as positive. Three slices were randomly selected, and 100 cells were screened for on each slice, which is located from the upper, lower, left, right and central visual fields, respectively. P27kipl and Ki67 positive cells that accounted for less than 10% of the total cell number of view was negative (cyclin E was 5%), 10-50% positive (cyclin E was 5-50%), and more than 50% was strongly positive.

RT-PCR method was applied to detect corresponding expression levels of mRNA, and the total RNA extraction kit was purchased from the R&D Systems (Inc., Minneapolis, MN, USA). Ultraviolet spectrophotometry (NanoDrop Spectrophotometer ND-1000, Thermo Fisher, Waltham, MA, USA) was used to measure RNA absorbance and the purity and concentration of detected RNA. The integrity of RNA was tested by agarose gel (Beijing Jinboyi Biological Technology Co. Ltd., Beijing, China) electrophoresis method. PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, Shenyang, China) was used for reverse transcription to synthesize cDNA. Primer was synthesized by Shanghai Boshang Biological Technology (CO. Ltd., Shanghai, China) with the application of Power SYBY Green PCR Master Mix (ABI, Carlsbad, CA, USA) reaction system, including Power SYBY Green PGR MasterMix 5 μl, each 0.5 μl of forward primer and reverse primer, ddH2O 2 μl and cDNA template 2 μl. The reaction condition was 95°C 5 min, 95°C 20s, 60°C 60s and 40°C 20s, a total for 45 cycles, with 95°C 15s and 60°C 60s, melting curve, 95°C 15s until the end. The result was expressed as $2^{-\Delta\Delta Ct}$.

This study was completed by the same surgical and nursing team, according to a standard medical procedure. For patients who have indications for surgical resection, the open or radical laparoscopic resection of the tumor was used. The palliative surgery was used in patients with advanced stage resection of lesions, which was combined with postoperative radiotherapy and chemotherapy regimen of FOLFOX4, which lasted for at least for six cycles. The relativity of expression level of Cyclin E, p27kipl and Ki-67 in tissues and TNM staging, pathological types, free progression survival and median survival was analyzed. The sensitivity, specificity, and accuracy of diagnosis were analyzed by receiver operating curve (ROC).

Statistical Analysis

SPSS20.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis. Measurement data was expressed as mean±standard deviation. The independent samples t-test was used to compare the two groups. Count data was expressed by case or (%), and the χ^2-test was adopted in the comparison between groups. Rank sum test was used to compare rank data. The survival used Kaplan-Meier model, with log-rank χ^2-test; ROC curve analysis was applied for the sensitivity, specificity and critical value of diagnosis, and the accuracy was expressed by an area under the curve (AUC). $p<0.05$ indicated statistical significance.

Results

Comparison of Positive Expression Rate of Cyclin E, p27kipl and Ki-67 in Tissues

Positive expression rate and positive degree of Cyclin E and Ki-67 in tissues of observation group were higher than those of the control group, while positive expression rate and positive degree of p27kipl was lower than that of the control group; the difference was statistically significant ($p<0.05$). (Table I).

Comparison of Quantitative Expression Level of Cyclin E, p27kipl and Ki-67 mRNA in Tissues

The quantitative expression levels of Cyclin E and Ki-67 mRNA in tissues of the observation group were distinctly higher than those of the control group, while p27kipl levels were evident-

Table I. Comparison of positive expression rate of Cyclin E, p27kipl and Ki-67 in tissues [n(%)].

Groups	n	Cyclin E			p27kipl (decrease)			Ki-67		
		Negative	Positive	Strong positive	Positive rate	Negative	Positive	Strong positive	Positive rate	Negative	Positive	Strong positive	Positive rate
Control group	200	180	18	2	20 (10.0)	30	98	72	170 (85.0)	178	19	3	22 (11.0)
Observation group	200	35	105	60	165 (82.5)	175	21	4	25 (12.5)	42	88	70	158 (79.0)
χ^2		213.585	211.439	213.227	210.381	190.061	186.828						
p		0.000	0.000	0.000	0.000	0.000	0.000						

Table II. Comparison of quantitative expression level of Cyclin E, p27kipl and Ki-67 mRNA in tissues.

Groups	Cyclin E	p27kipl	Ki-67
Control group	0.0359±0.0028	0.6487±0.0287	0.0528±0.0048
Observation group	0.6324±0.0164	0.0467±0.0063	0.6128±0.0349
t	164.527	148.273	186.493
p	0.000	0.000	0.000

ly lower than that of the control group, and the difference was statistically significant ($p<0.05$). (Table II)

The Relativity of Positive Expression of Cyclin E, p27kipl and Ki-67 in tissues and TNM Staging, Pathologic type of Observation Group

With the increase of TNM staging, the positive expression of Cyclin E and Ki-67 in tissues was increased, p27kipl decreased, and the difference was statistically significant ($p<0.05$). With the decrease of differentiation degree, the positive expression of Cyclin E and Ki-67 increased, p27kipl decreased, and the difference was statistically significant ($p<0.05$). (Table III).

The Relativity of pPositive Expression of Cyclin E, p27kipl and Ki-67 in Tissues and free Progression Survival and Median Survival

The free progression survival and median survival of positive expression and negative expression of Cyclin E and Ki-67 in tissues were shortened, p27kipl extended, and the difference was statistically significant ($p<0.05$). (Table IV).

ROC Curve Analysis

The expression levels of Cyclin E, p27kipl and Ki-67 mRNA of the control group and observation group were regarded as diagnostic factors, and colorectal cancer as the diagnostic result, which were included into ROC model for analysis. Results showed that the diagnostic sensitivity of Cyclin mRNA was 89.6%, specificity 84.5%, accuracy 0.823, 95%CI=0.658-0.965, $p=0.021$, and critical value was 0.3562. The diagnostic sensitivity of p27kipl mRNA was 80.5%, specificity 76.5%, accuracy 0.802, 95% CI=0.613-0.925, p=0.028, and critical value was 0.3023. The diagnostic sensitivity of Ki-67 mRNA was 86.5%, specificity 82.9%, accuracy 0.814, 95%CI=0.633-0.942, $p=0.023$ and critical value was 0.3243 (Figure 1).

Table III. The relativity of positive expression of Cyclin E, p27kipl and Ki-67 in tissues and TNM staging, pathologic type [n(%)].

	Cyclin E (n=165)	p27kipl (n=25)	Ki-67 (n=158)
Stage I (n=52)	30 (57.7)	13 (25.0)	32 (61.5)
Stage II (n=43)	38 (88.4)	7 (16.3)	33 (76.7)
Stage III (n=60)	55 (91.7)	4 (6.7)	53 (88.3)
Stage IV (n=45)	42 (93.3)	1 (2.2)	40 (88.9)
χ^2	30.343	26.833	15.492
p	0.000	0.000	0.001

Table IV. The relativity of positive expression of Cyclin E, p27kipl and Ki-67 in tissues and free progression survival and median survival.

	Cyclin E		p27kipl		Ki-67	
	Negative (n=35)	Positive (n=165)	Negative (n=175)	Positive (n=25)	Negative (n=42)	Positive (n=158)
Free progression survival (month)	16.6	9.5#	10.2	17.0#	15.7	9.2#
Median survival (month)	32.7	25.5#	32.6	42.5#	36.5	28.7#

Note: #, free progression survival and median survival of positive expression and negative expression of Cyclin E, p27kipl and Ki-67 in tissues, $p<0.05$.

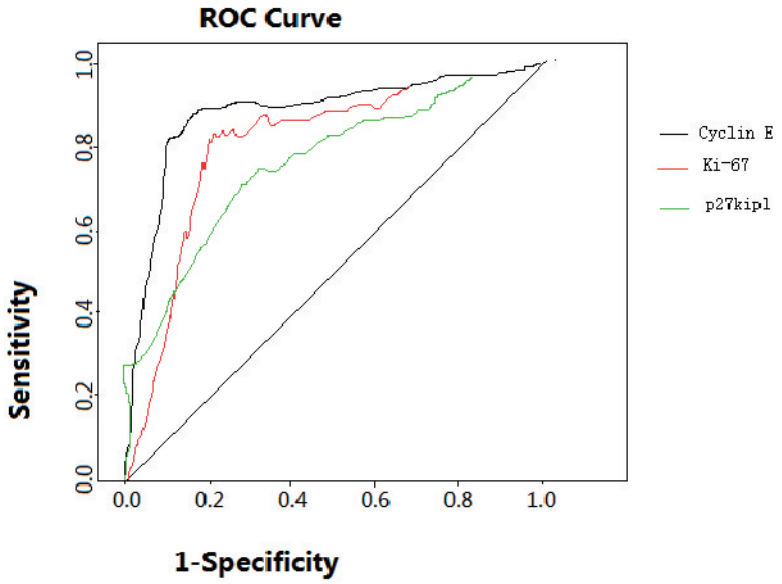

Figure 1. ROC diagnostic analysis of Cyclin E, p27kipl and Ki-67 mRNA level in tissues.

Discussion

According to different morphological and biochemical changes, the cell cycle was divided into four stages: G1 (DNA pre-synthesis phase), S (DNA synthesis phase), G2 (DNA post-synthesis phase) and M (mitotic phase). Whether cells entered the division cycle and the division cycle can be successfully completed depended on whether cells can go through several checkpoints; of those, the most important are G1/S and G2/M[8]. The former is referred as the Start point or Restriction point, which locates to the end of the G1 phase and is the starting point of the cell cycle; the latter locates at the start of M phase. G1 is the only phase where proliferating cells can accept external proliferation and proliferation inhibition signals. The events that regulate the proliferation and differentiation of cells mainly occur during the G1 phase and their regulation disorder has significance in the occurrence and development of tumors[9].

The activated CDKs have important regulating effects in the conversion process of G1/S and G2/M. Moreover, its activity directly affects the change of the cell cycle[10]. The abnormal increase in CDK activity can make cells appear to be a non-inhibition in growth, to form tumors, and therefore, the over-expression of CDKs enhancing factor or deficiency or lack of expression of inhibitory factor can lead to uncontrolled cell proliferation and the formation of tumors[11]. Cyclin E is a positive regulatory factor of CDKs, which has positive regulatory effects of the cell cycle[12]. CDKIs can inhibit the activity of CDKs, which has negative regulatory effects[13]. As the important member of CDKIs family, p27kipl has strong inhibition of histone H1 kinase-like activity and pRb phosphorylation activity of Cyclin A-CDK2 and Cyclin E-CDK2, which can make cell block in G1 phase and inhibit cell proliferation, so as to exert the negative regulatory effects of the cell cycle[14].

The study indicated that the positive expression rate and positive degree of Cyclin E and Ki-67 in tissues of observation group were higher than those of the control group, while the positive expression rate and positive degree of p27kipl were lower than that of the control group; the difference was statistically significant. The quantitative expression levels of Cyclin E and Ki-67 mRNA in tissues of observation group were distinctly higher than those of the control group, while p27kipl was evidently lower than that of the control group; the differences were statistically significant, which implied that the high expression of Cyclin E and Ki-67, and the low expression of p27kipl are closely associated with the occurrence of colorectal cancer. With the increase of TNM staging, the positive expression of Cyclin E and Ki-67 increased, p27kipl decreased, and the difference was statistically significant. With the decrease of differentiation degree, the positive expression of Cyclin E and Ki-67 increased, p27kipl decreased, and the difference

was statistically significant, which implied that Cyclin E, p27kipl and Ki67 protein were closely related to the proliferation, differentiation and invasive biological behavior of the tumor. The free progression survival and median survival of positive expression and negative expression of Cyclin E and Ki-67 shortened, p27kipl extended, and the difference was statistically significant. It implied that Cyclin E, p27kipl and Ki67 protein were closely associated with therapeutic effect and long-term prognosis of the tumor, which may be intrinsic biological molecules that have a role in regulating tumor properties. The diagnostic sensitivity of Cyclin E mRNA was 89.6%, specificity 84.5%, accuracy 0.823 and the critical value was 0.3562. The diagnostic sensitivity of p27kipl mRNA was 80.5%, specificity 76.5%, accuracy 0.802 and the critical value was 0.3023. The diagnostic sensitivity of Ki-67 mRNA was 86.5%, specificity 82.9%, accuracy 0.814 and the critical value was 0.3243. It implied that Cyclin E, p27kipl and Ki67 could be used as highly sensitive, specific and accurate indexes for the diagnosis of colorectal cancer.

Conclusions

We suggest that Cyclin E, p27kipl and Ki67 in tissues can be used as new targets for early intervention of tumor as well as significant application value for improving clinical effects and long-term.

Conflicts of interest

The authors declare no conflicts of interest.

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